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16. ABSTRACT

The time required for the microbially mediated dechlorination of chloroaromatics in anaerobic freshwater sediments may be dependent upon sediment microbial communities and physical characteristics, as well as chlorine position on the aromatic ring. To better understand such possible relationships, we studied the reductive dechlorination of three dichlorophenol isomers in anaerobic sediments collected every other month for a year from five sites in one pond. Numbers of specific dechlorinating microorganisms were estimated by a most probable number technique, and sediment temperature, pH, Eh, and organic carbon content were determined. Levels of dissolved organic carbon, sulfate, and nitrate also were quantified in water samples collected with the anaerobic sediments. The relative susceptibility of the 2,4-, 2,5-, and 3,4- isomers to dechlorination was determined in sediment slurries. Dechlorination to monochlorophenols required from 2 to more than 32 weeks for completion, with the relative rates being 2,4->2,5->3,4-. The number of specific dechlorinating microbes appears to be important in determining the time required for this transformation.

17. KEY WORDS AND DOCUMENT ANALYSIS

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A. Abstract.

The time required for the microbially mediated dechlorination of chloroaromatics in anaerobic freshwater sediments may be dependent upon sediment microbial communities and physical characteristics, as well as chlorine position on the aromatic ring. To better understand such possible relationships, we studied the reductive dechlorination of three dichlorophenol isomers in anaerobic sediments collected every other month for a year from five sites in one pond. Numbers of specific dechlorinating microorganisms were estimated by a most probable number technique, and sediment temperature, pH, E_h, and organic carbon content were determined. Levels of dissolved organic carbon, sulfate, and nitrate were also quantified in water samples collected with the anaerobic sediments. The relative susceptibility of the 2,4-, 2,5-, and 3,4- isomers to dechlorination was determined in sediment slurries. Dechlorination to monochlorophenols required from 2 to more than 32 weeks for completion, with the relative rates being 2,4->, 2,5->, 3,4-. The number of specific dechlorinating microbes appears to be important in determining the time required for this transformation.

B. Introduction.

A number of aliphatic and aromatic compounds, including xenobiotics, are biotransformed anaerobically[1-10]. Within the past decade, significant progress has been made in understanding the anaerobic biotransformations of organics. Initially, such transformation processes were studied using pure or mixed

anaerobic cultures, including photosynthetic, respiratory, and fermentative microorganisms. More recently the biotransformation of a variety of organic compounds has been studied in samples from several anaerobic sediments, soils, aquifer materials, and sewage sludges.

Chlorinated phenols, which are listed as Priority Pollutants by the U.S. Environmental Protection Agency[11], are biodegraded in a number of anaerobic ecosystems[8,12-15]. The initial transformation in the anaerobic biodegradation of chlorophenols is often reductive dechlorination to phenol. Phenol may then be degraded either through cyclohexanol, cyclohexanone, and adipate to succinate, propionate, and acetate[9,10] or through benzoate and acetate to the end products, carbon dioxide and methane.[16-18]

Much of the early work has concentrated on the degradation pathways of chlorinated phenols, with future work to be directed toward understanding the environmental factors and chemical characteristics that influence the fate of these compounds in anaerobic ecosystems. In this chapter, we present results of experiments designed to examine the effects of environmental parameters on the reductive dechlorination of chlorinated phenols. Research in our laboratory has focused on this reaction because reductive dechlorination is often the initial step, and possibly the rate-limiting step[18], in the anaerobic degradation of these compounds.

C. Initial Research.

To determine the prevalence and specificity of microbial dechlorinating activity in anoxic freshwater sediments, we initially studied the reductive dechlorination of the six dichlorophenol (DCP) isomers (2,3-, 2,4-, 2,5-, 2,6-, 3,4-, and 3,5-) in sediments from five ponds in the Athens, Georgia, area[19]. Although dechlorination of several isomers was evident in sediments from each of the ponds, a range of from 20 days to more than 98 days was required for transformation of 50% of a dichlorophenol to a monochlorophenol (Table 1). Sediment microorganisms in Cherokee Pond exhibited the highest dechlorinating activity, as evidenced by T_{50} values for the DCPs of from 20 to 35 days. The 3,4- isomer was an exception with a T_{50} of >84 days. In contrast, dechlorinators in the other four pond sediments were less active toward the DCPs, as reflected in the longer T_{50} values for each of the isomers. In the five sediments, chlorines were removed from the dichlorophenols in the order *ortho* > *meta* > *para*. A similar order of dechlorination was determined for the monochlorophenol products.

Characterization of the dechlorinating activity present in the most active sediment (Cherokee Pond) and in a less active sediment (Bolton's Pond) was undertaken to examine the disparity in dechlorinating activity of the pond sediments[14]. The dechlorinating activity and substrate specificity of the sediment microbes were determined first by repeated exposure of sediment slurries to 2,3-, 2,4-, or 2,6-DCP. Subsequently, subsamples of the acclimated sediment slurries were individually exposed to each of the six DCP isomers to determine substrate specificity.

Table 1. Persistence of dichlorophenols in anaerobic sediment slurries from five ponds[a].

Dichlorophenol	T_{50} (days)[b] Pond Sediment: Cherokee	Bolton's	Bar H	Sandy Creek	2-Boat
2,3-	20 (17)[c]	34 (98)	36	39	29
2,4-	21 (20)	21 (56)	21	21	21
2,5-	33	47	>98	61	>98
2,6-	34 (21)	>98 (224)	>98	>98	>98
3,4-	>84	>84	>84	49	>84
3,5-	35	42	>84	48	21

[a]Samples of sediment (0-10 cm) and overlying water were collected from the five ponds. The samples, which were collected in sterile wide-mouth quart Mason jars and Erlenmeyer flasks, respectively, were transported to the laboratory and placed into an anaerobic chamber with an atmosphere 95% N_2:5% H_2. The sediments were sieved and stored as previously described[14]. Twenty-milliliter slurries (1:10 weight:volume) were prepared in 100-ml serum bottles, which were capped with butyl rubber stoppers and crimp sealed. Assays were initiated by addition of 1 milliliter of an aqueous stock solution (200 mg/L) of a dichlorophenol to the reaction vessels to yield a final concentration of 10 mg/L. Incubation of serum bottles and high pressure liquid chromatography of slurry extracts followed a previously described protocol.[14]

[b]Numbers represent the time for dechlorination of 50% of a dichlorophenol to a monochlorophenol.

[c]Numbers in parentheses represent the T_{50} for a specified dichlorophenol in sediments from Cherokee and Bolton's Ponds in a second study of microbial dechlorinating activity[14].

A period of acclimation was observed in sediments from both ponds upon initial exposure to a DCP. No significant loss of dichlorophenol or production of monochlorophenol occurred during this time. The acclimation periods, ranging from 12 to 14 days in Cherokee sediments and from 35 to 196 days in Bolton's sediments, were similar to those observed in the initial study of five pond sediments[19]. Acclimation periods were not detectable following a second addition of dichlorophenol. Repeated addition of 2,3-, 2,4-, or 2,6-DCP to sediments acclimated the indigenous microorganisms to initially remove the *ortho* chlorine without a lag. Acclimated Cherokee sediments exhibited faster dechlorination rates and a broader substrate specificity (data not shown) than those in acclimated Bolton's sediments.

D. Dechlorination of 2,4-, 2,5-, and 3,4-Dichlorophenol in Cherokee Pond Sediment Slurries.

Because sediment characteristics may be expected to vary temporally and spatially, a study was initiated to determine selected characteristics and microbial dechlorinating activities of sediments collected every other month for a year from five sites within Cherokee Pond (Fig. 1). Experiments were designed to determine correlations between selected sediment properties and rates of dechlorination of 2,4-, 2,5-, and 3,4- DCP. Our objective was to utilize the correlations to predict the persistence of chlorophenols with various chlorine substitution patterns in anoxic sediments.

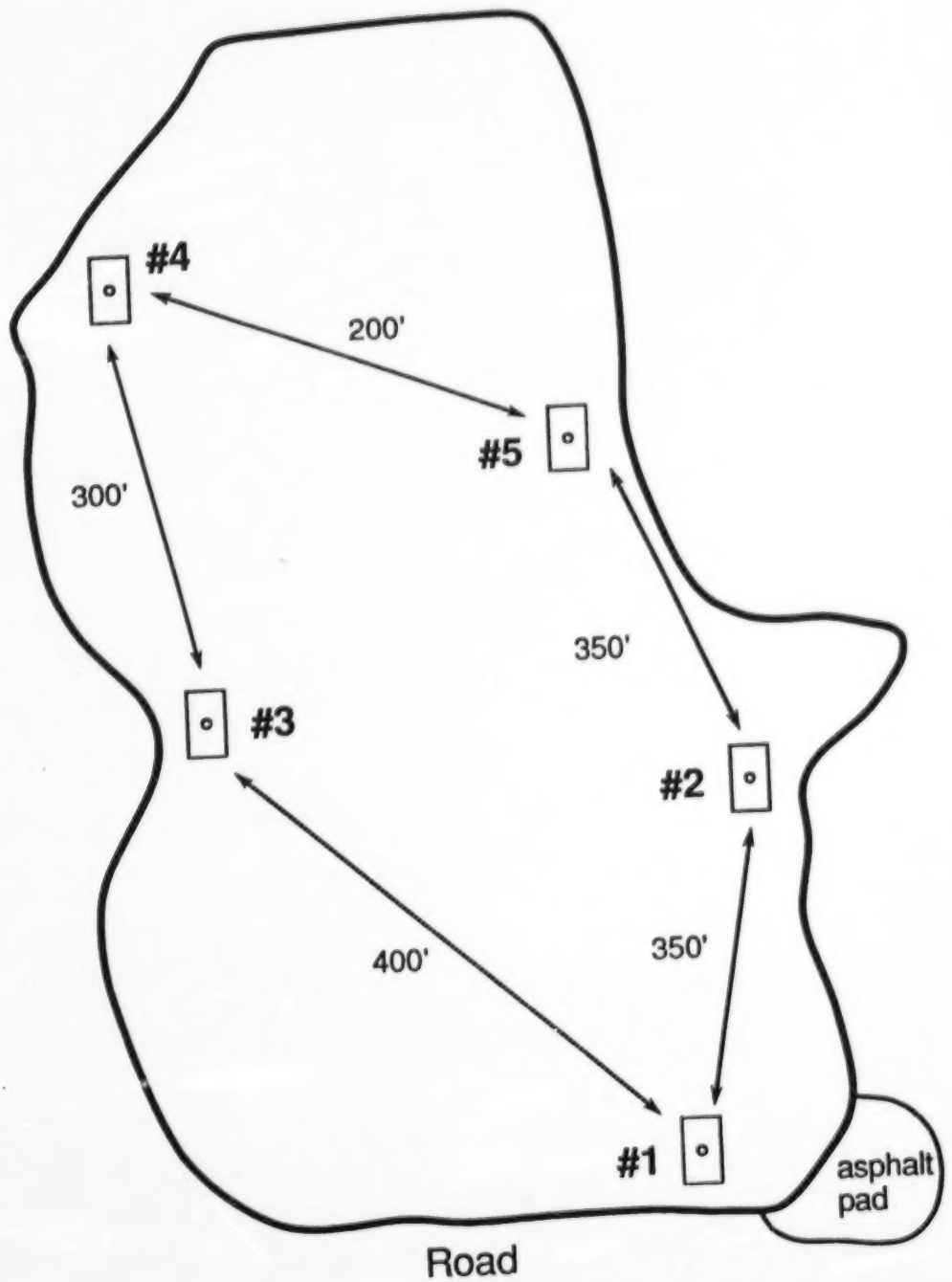

Figure 1. Location of the five sediment sampling areas (10 x 30 ft) in Cherokee Pond.

The time required for reductive dechlorination of 2,4-, 2,5- and 3,4-DCP to monochlorophenols varied temporally and spatially in Cherokee sediments collected every other month between May 1988 and May 1989. Data for sites 1, 3, and 4 are summarized in Table 2. Microorganisms in sediments from these sites exhibited low (site 1), high (site 4), and intermediate (site 3) dechlorinating activity. Dechlorinators in sediments from sites 2 and 5 exhibited activities similar to those determined in site 3.

No seasonal pattern for dechlorination of the three isomers was evident from the data. With the exception of T_{50} values for the DCPs in July and November 1988 sediments (and in some January 1989 sediments), mean T_{50} values for each of the isomers ranged from 6 to 30 days in sediments collected from all sites. Mean T_{50} values for DCPs in sediments from each site except site 4 were elevated in July and November.

Significant differences in the T_{50} values of the three DCPs were observed in sediments from different sites. The mean T_{50} value for each isomer was highest in sediments from site 1, averaging 64, 63, and 74 days for the 2,4-, 2,5-, and 3,4- isomers, respectively. Conversely, the mean T_{50} for each isomer was lowest in sediments from site 4. The 2,4- and 2,5-DCPs were dechlorinated more rapidly (T_{50}=13 and 14 days, respectively) than 3,4-DCP (T_{50}=23 days) in sediments from this site. A similar susceptibility to dechlorination was observed for 2,4-, 2,5-, and 3,4-DCP in sediments from site 3, with mean T_{50} values of 25, 38, and 56 days, respectively. Thus, a range of T_{50} values was observed for each isomer in Cherokee sediments from different sites.

Table 2. Persistence of 2,4-, 2,5-, and 3,4-dichlorophenol in Cherokee sediment slurries.[a]

T_{50} (days)[b]

Sampling Date	Sampling Site and Dichlorophenol Isomer								
	1			3			4		
	2,4-	2,5-	3,4-	2,4-	2,5-	3,4-	2,4-	2,5-	3,4-
May	24	26	25	21	20	32	21	22	25
July	192	173	160	88	98	93	12	17	19
September	72	17	29	11	15	20	12	18	20
November	91	155	159	25	92	139	19	14	21
January	10	14	66	7	15	64	10	14	20
March	43	41	50	7	7	6	7	7	9
May	19	16	32	16	21	35	12	9	45

[a]Samples of sediment (0-10 cm) and overlying water were collected every other month for a year from selected points in the five sites chosen for study in Cherokee Pond. The samples were treated as described in Table A, and assays were initiated by addition of 1 milliliter of an aqueous stock solution (200 mg/L) of 2,4-, 2,5-, or 3,4- dichlorophenol to the reaction vessels to yield a final concentration of 10 mg/L. Incubation of serum bottles (including control and autoclaved reaction vessels) and high pressure liquid chromatography of slurry extracts followed a previously described protocol.[14]

[b]Numbers represent the mean time (n=3) for dechlorination of 50% of the respective dichlorophenol to a monochlorophenol in three different sediments from the same site.

In considering the influence of chlorine substitution on DCP dechlorination, a regiospecific pattern was observed for the initial dechlorination of each isomer. As was previously observed in the study of five pond sediments[19], the 2,4- and 2,5- isomers were reductively dechlorinated to 4- and 3-chlorophenol (CP), respectively, by removal of the *ortho* chlorine, and the 3,4- isomer was dechlorinated at the *para* position to form 3-CP. Dechlorination at the *meta* position of the 3,4- isomer was detected only once in the 105 sediment slurries examined, and in this case, 3-CP and 4-CP were produced in approximately equal amounts.

E. Sediment/Water Characteristics and Their Correlation to Dichlorophenol Dechlorination.

In order to determine any correlation between the T_{50} values for the DCPs and properties of the sediment/water system, the Cherokee sediment and water samples were analyzed for several physical, chemical, and microbiological characteristics. These included in situ temperature, pH, redox potential (E_h), sediment organic carbon, dissolved organic carbon, nitrate concentration, sulfate concentration, and the number of 2,4-DCP dechlorinating microorganisms. Tables 3 and 4 summarize data on sediment pH, sulfate and nitrate concentration in site water, and number of 2,4-DCP dechlorinating sediment microorganisms of samples from sites 1, 3, and 4.

Some sediment/water characteristics displayed no significant temporal and/or spatial variations. Sediment temperatures ranged from 10 to 30°C

during the sampling year but did not vary from one site to another by more than 0.5°C at any one sampling time. Similarly, water temperatures, which paralleled those of sediments from the same site, varied significantly temporally but not spatially. Mean sediment redox potentials fluctuated during the study period from -125 mV (May 1988) to -4 mV (September 1988). However, spatial variability was limited, as evidenced by the mean E_h range of -32 to -59 mV for sediments from all sites. Sediment redox potentials were highest (+130 mV) in May 1989 when the water column was restored after a 2-month period during which the pond was drained.

Sediment/water characteristics that displayed significant temporal and/or spatial variation were pH, organic carbon content, nitrate and sulfate concentration, and the number of 2,4-DCP dechlorinating microorganisms. The mean pH of sediments from all sites ranged from 5.3 to 7.1 during the study period. Extremely acidic pH's (i.e., 4.7) were reported for May 1989 sediments, which were collected after the water column was restored to the drained pond (Table 3). The organic carbon content of Cherokee sediments and water varied to a greater extent temporally than spatially (data not shown). During the study year, sediment organic carbon averaged 0.1 to 2.0%. The highest average concentrations of total organic carbon (TOC) occurred in sediments collected in September (0.68%) and November 1988 (0.92%) and in March 1989 (1.1%). Similar peaks in sediment TOC over time were not observed on a site basis. Mean TOC was highest in site 4 sediments (0.89%) and lowest in site 1 sediments (0.48%). Dissolved organic carbon, which was often less than 10 ppm, also varied significantly over time but not with site (data not shown).

Table 3. Sediment pH[a] and sulfate[b] and nitrate[c] concentrations (ppm) in water from three sites in Cherokee Pond.

Collection Date	Site 1			Site 3			Site 4		
	pH	SO_4	NO_3	pH	SO_4	NO_3	pH	SO_4	NO_3
May	6.4-6.6	0.23	ND*	6.3-6.6	0.17	ND	6.4-6.6	0.20	ND
July	6.3-6.7	0.36	ND	6.4-6.7	0.19	ND	6.4-6.5	0.58	ND
September	5.8-6.1	0.77	0.73	6.0-6.1	1.11	0.71	6.0-6.2	0.76	0.72
November	6.5-6.8	0.60	1.47	6.2-6.6	0.62	1.54	6.0-6.2	0.53	1.49
January	5.9-6.0	0.37	ND	6.0-7.0	0.90	ND	6.5	0.08	ND
March	5.8	NW**	NW	7.0	NW	NW	6.7	NW	NW
May	4.7-5.4	12.48	ND	5.1-5.7	11.73	ND	5.4-6.6	0.36	ND

*Not detectable
**No water column (pond drained)

[a]Numbers represent the range of pH determined for three sediments from each site. Determinations were made with an Orion Research digital pH/millivolt meter (Model 611) with a combination pH electrode (Orion 977800). The electrode was calibrated with pH 4 (potassium biphthalate) and pH 7 (potassium phosphate monobasic-sodium hydroxide) buffers (Fisher Scientific).

[b,c]Sulfate and nitrate concentrations were determined with a Dionex 2020i ion chromatograph utilizing an HPIC AS-4 analytical column and electrometer. The standard eluent, run at 1.5 ml/min, was 0.003 M $NaHCO_3$/0.0024 M Na_2CO_3. A 0.025 N H_2SO_4 solution was used with the continuous flow fiber suppressor. Water samples were injected through a 0.22-μ filter onto a 100-μ loop. A Hewlett-Packard 3390A integrator was used to quantify sulfate and nitrate by comparison to Na_2SO_4 and KNO_3 standards, respectively. Reported concentrations are the mean of triplicate determinations of water samples from each of three locations within each site.

The concentration of nitrate and sulfate in site water varied significantly over time and, in some instances, with site (Table 3). From September to November 1988, nitrate levels doubled from a mean of 0.72 ppm to a mean of 1.51 ppm. However, nitrate concentrations were not significantly different from site to site. With the exception of September 1988 and May 1989, at every sampling time the average sulfate concentration in water from each site was less than 1 ppm. During the year, sulfate concentrations increased in water samples collected from May through September but generally decreased in water samples collected from September through January. The highest concentrations of sulfate, which occurred in the May water samples from sites 1 (12.48 ppm) and 2 (11.73 ppm), coincided with the restoration of the water column following drainage of the pond. Why a similar peak in sulfate concentration was not observed in water from site 4 is not clear. However, over the study period, the mean sulfate concentration (0.42 ppm) was lowest in water from this site.

The number of 2,4-DCP dechlorinating microorganisms in Cherokee sediments varied temporally and spatially (Table 4). At any one sampling time (except January 1989) or site, the number of these organisms in sediments varied from one to two orders of magnitude. Most probable numbers (MPN) values of dechlorinators in most sediments increased from September to January and from January to March and decreased from March to May. Drainage of the pond may have affected MPN values of dechlorinators in sediments collected in May.

A statistical analysis of the data indicated that certain sediment characteristics may be used to predict the T_{50} values of the DCPs. For each of the isomers, a multiple regression analysis was performed using data from

Table 4. Most probable number (MPN) of 2,4-dichlorophenol dechlorinating microorganisms in Cherokee sediments.[a]

MPNs (x 10^3 g^{-1} dry sediment weight)

Sediment Collection Date	Sediment Collection Site 1	3	4
September	0.18	0.52	33.0
January	4.6	<2.9	4.6
March	<8.5	19.0	48.0
May	<2.9	48.0	<2.9

[a]Numbers of 2,4-dichlorophenol dechlorinating microorganisms were estimated by a dilution procedure utilizing filter-sterilized (0.22 μ), autoclaved (15 psi, 30 minutes) site water. Dilutions of 10^{-1} to 10^{-5} were prepared by serial dilution of 0.5 ml of slurry into 4.5 ml of sterile site water. Subsequent transfer of 0.5 ml of these dilutions to 4.5-ml volumes of sterile water with 2,4-dichlorophenol yielded final slurry dilutions of 10^{-2} to 10^{-6} with 10 mg/L 2,4-dichlorophenol per tube. Triplicate tubes were prepared at each dilution and incubated for 1 month before acetonitrile extracts of slurry dilutions were analyzed by a previously described HPLC protocol.[14] Tubes with a chromatogram having a 2,4-dichlorophenol peak area not more than three times the peak area of the 4-chlorophenol were scored positive. Numbers of dechlorinators were estimated from the number of positive tubes in consecutive dilutions using an MS-DOS turbo pascal program using the American Society for Microbiology guidelines for MPN determinations.

all sites and sampling dates. For the 2,5- and 3,4- isomers, the variables of pH and nitrate and sulfate concentrations (as well as redox potential for 2,5-DCP) accounted for 82% of the variation in T_{50} values. Approximately 86% of the variation in T_{50} values of these isomers was explained by using these variables in addition to those of redox potential, the ratio of carbon dioxide to hydrogen in the headspace of the test vessels (data not shown), and sediment organic carbon. However, this 4% increase in accounting for T_{50} variations does not constitute a significant contribution to the regression equation. For 2,4-DCP, regression analysis of data from all sites and sampling times indicated that none of the variables (alone or in combination) accounted for more than approximately 50% of the variation in the T_{50} values. Further research is needed to identify other sediment characteristics that may be used to predict variations in the T_{50} values of 2,4-DCP and the reasons that it is different from the other isomers. Additional studies are also necessary to determine the utility of this model in predicting the T_{50} values of the DCPs in sediments from other ponds.

The use of the variables of pH, nitrate and sulfate concentration, and E_h to predict the T_{50} values of selected DCPs (i.e., 2,5- and 3,4-) in anoxic sediment/water systems has practical implications. These characteristics can be determined rapidly with pH, ion selective, and E_h electrodes. Levels of the anions also may be determined by use of ion chromatography. The data may then be used in regression equations to predict T_{50} values of specified chlorophenols.

The results of the present study indicate that the reductive dechlorination of chlorinated phenols in anaerobic sediments may be correlated to sediment/water properties. In particular, the pH, nitrate and sulfate

concentrations, and E_h of sediment/water systems may be used to predict the persistence of selected DCPs, with chlorines removed from the ring in the order *ortho* > *meta* or *para*. Although regression analysis did not indicate that the number of dechlorinating microorganisms present in the sediments at the time of dichlorophenol addition could be used to predict T_{50} values, ongoing experiments indicate that a "threshold" number of dechlorinators may be necessary before loss of the parent compound is detectable. Future research will hopefully provide information on the metabolism and growth of these dechlorinating microorganisms in natural anoxic environments. A better understanding of the way in which environmental conditions and chemical structure influence chlorophenol degradation should result.

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NOTE: Mention of trade names or commercial products does not constitute endorsement or recommendation for use by the U.S. Environmental Protection Agency.

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